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Viral lysis of bacteria: an important source of dissolved amino acids and cell wall compounds

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Viral infection of bacteria causes release of dissolved organic matter (DOM), which is available for bacterial uptake. In aquatic environments, this virus-mediated transformation of living cells into dissolved and colloidal organic matter may be a quantitatively important process in the pelagic recycling of carbon and nutrients, but little is known about the amount, composition, or bioavailability of viral lysates. By using a model system of a marine bacterium (*Cellulophaga* sp.) and a virus specific to this bacterium, the present study provides a first quantification of the input of dissolved free and combined amino acids (DFAA and DCAA) and bacterial cell wall compounds following viral lysis. The DCAA constituted 51–86% of the total virus-mediated organic carbon release of 1087–1825 $\mu\text{g C l}^{-1}$ (estimated biomass of the lysed bacteria), whereas DFAA and glucosamine each accounted for 2–3% of total lysate-C. The viral particles themselves constituted 4–6% of the released organic carbon, and altogether, the applied analyses thus identified 53–92% of the released lysates. Approximately 12% of the identified compounds were derived from bacterial cell wall peptidoglycan, including various D-isomers of DFAA and DCAA, glucosamine and diaminopimelic acid (DAPA). Although a portion of this cell wall material may have entered the pool of refractory material, a significant fraction of some peptidoglycan-derived components, e.g. 83% of the released D-DFAA, were removed from the dissolved phase during the last part of the incubations, suggesting that part of the cell wall material were utilized by the developing virus-resistant *Cellulophaga* population. Therefore, we suggest that virus-mediated DOM is a source of a variety of organic compounds, which contribute significantly to the pool of rapidly recycling material in the ocean.

INTRODUCTION

Infection of bacteria by lytic viruses leads not only to production of new viruses; it also causes the release of dissolved organic matter (DOM) as the bacterial cells disrupt. These viral cell lysates are potentially labile compounds that can be utilized by other bacteria. This means that the virus-mediated regeneration of DOM may affect the cycling of carbon and nutrients. In marine systems it is assumed that 20–40% of the bacteria are killed by viruses on a daily basis (Suttle, 2005) transforming bacterial biomass to DOM. This virus-mediated DOM release equals a production of approximately 3–20 Gt dissolved organic carbon per year on a global scale (Wilhelm & Suttle, 1999). Considering that global phytoplankton primary production is approximately 50 Gt C y⁻¹, it appears that viral lysis of bacteria is a quantitatively important process in the marine carbon cycle.

The fate of viral lysates in aquatic environments depends on whether they can be assimilated by bacteria. In contrast to most ambient DOM in seawater, freshly released DOM from lysed cells is believed to be labile for bacterial consumption (Amon & Benner, 1996). A close coupling between release and subsequent uptake of viral lysates was demonstrated by Middelboe et al. (2003), who observed that 28% of viral lysates was converted to new biomass of non-infected bacteria within two days after

cell lysis. This confirmed that a large portion of the lysates can be readily assimilated by bacteria. The study illustrated that virus-induced substrate transformation provided utilizable substrate (the lysates) for a bacterial type that was unable to grow on the bulk DOM pool.

Bioavailability of viral lysates depends on their chemical and structural composition. Shibata et al. (1997) identified peaks of sub-micron sized particles (0.38–0.7 μm) following viral lysis of *Vibrio alginolyticus*, and suggested that these particles were cell debris originating from bacteria. The chemical composition of the dissolved fraction (<0.2 μm) of viral lysates is, however, virtually unknown. Regeneration of DOM following viral lysis of marine bacteria and microalgae has been shown to include nutrients such as P (e.g. Middelboe et al., 1996; Noble & Fuhrman, 1999) and N, Fe and Se (Gobler et al., 1997; Poorvin et al., 2004), which then became available to bacteria or phytoplankton through viral activity. The efficient utilization of viral lysates and subsequent stimulation of bacterial and phytoplankton growth, suggests that viral lysates indeed may play an important role in pelagic nutrient cycling.

Production of lysates and their bacterial cycling have typically been studied by isotope techniques or by changes in DOM pools (Noble & Fuhrman, 1999; Middelboe et al., 2003), but released bacteria-specific compounds can also be used as markers of cell lysis. Among such compounds are D-isomers of amino acids

and diaminopimelic acid (DAPA) that both occur in the peptide interbridges of cell wall peptidoglycan of bacteria. N-acetyl-glucosamine (located in the glycan backbone of peptidoglycan) has also been used as a marker of cell wall material (e.g. Jørgensen et al., 2003), but it should be considered that this compound is also a dominant component in chitin. In peptidoglycan, D-isomers have been found to constitute 5–15% of the total amino acid content (Jørgensen et al., 2003). In natural pools of dissolved combined and free amino acids (DCAA and DFAA), L isomers are most abundant and appear to reflect the composition in living cells (McCarthy et al., 1998). D-amino acid isomers typically make up a maximum of 10% of naturally occurring DFAA and DCAA (Jørgensen & Middelboe, unpublished data) and most likely originate from degradation of bacterial cell wall peptidoglycan (McCarthy et al., 1998). Since viral lysis of bacteria involves disruption of the cell walls, this lysis may potentially provide a substantial input of D-amino acids to the dissolved amino acid pool. In contrast, the content of amino acids in virus particles at typical concentrations in seawater seems to be insignificant relative to ambient pools of dissolved amino acids (Fuhrman, 1992; Kuznetsova et al., 2005).

Bacterial cell wall material has generally been regarded as recalcitrant material (e.g. Nagata et al., 2003). Using radiolabelled peptidoglycan from a Gram-negative bacterium, Nagata et al. (2003) observed that mineralization of peptidoglycan by natural marine assemblages was 2–21 times lower than that of bacterial proteins, and that polysaccharides in peptidoglycan were more recalcitrant than its peptide component. However, peptidoglycan D-amino acid isomers constitute a potential nutrient source to bacteria if other nutrients are low. This was shown by Perez et al. (2003) who observed an increased uptake of DAsp with water depth in the oligotrophic North Atlantic.

In the present study, we investigated changes in DOM composition following viral lysis of a bacterial population

in batch incubations, with particular focus on the contribution from bacterial cell wall peptidoglycan. The results demonstrated that viral lysates constitute a significant source of D-amino acids and glucosamine, and that the released cell products were partly recycled during incubations.

MATERIALS AND METHODS

Experimental set-up

A model system with the marine bacterium *Cellulophaga* sp. and a *Cellulophaga*-specific virus (Middelboe et al., 2003) was used to analyse changes in the DOM composition during viral lysis in batch cultures. The *Cellulophaga* sp. is a Gram-negative, yellow/orange pigmented strain that belongs to Cytophaga-Flavobacterium-Bacterioides group (Johansen et al., 1999). The specific strain used in the present study (Accession number AF497997 in GenBank) has an average cell volume of $0.52 \mu\text{m}^3$ when grown in glucose-enriched medium (below) (Middelboe et al., 2003). The growth medium was prepared as in Middelboe et al. (2003) and consisted of artificial seawater (70% GF/F filtered (Whatman) aged seawater and 30% distilled water), enriched with 0.52 mM NH_4Cl and 0.14 mM KH_2PO_4 , and pasteurized at 80°C for 40 min. Glucose (2 mM final concentration) was added as the sole organic carbon source. The medium was distributed in four 500 ml bottles and bacteria were inoculated to a density of approximately $2 \times 10^5 \text{ cells ml}^{-1}$. Two of the cultures received the *Cellulophaga*-specific virus (approximate density: $1 \times 10^5 \text{ virus particles ml}^{-1}$), while the other cultures served as control cultures without viruses.

Samples for microscopy of viruses and bacteria, and for analysis of amino acids, were collected at 7 to 11 hour intervals, except for the last two samplings (47 h interval). Samples for viral and bacterial abundances were fixed with 2% glutaraldehyde, whereas samples for amino acid

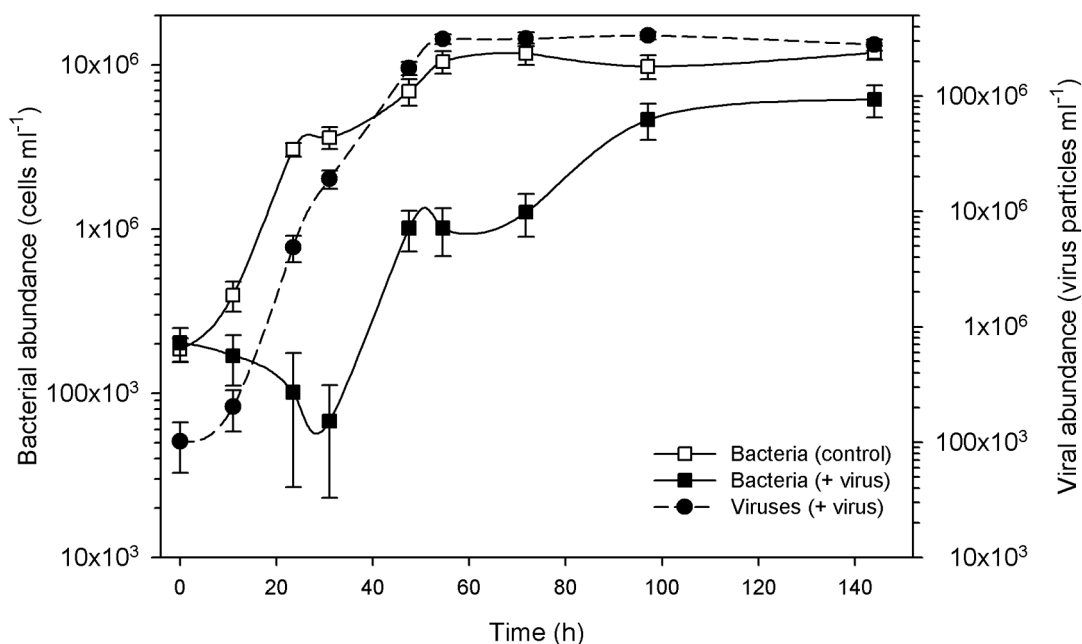


Figure 1. Abundance of bacteria and viruses in the control and virus-enriched (+virus) cultures. Average densities \pm SD estimated from 300 to 600 bacteria or viruses counted on each slide are shown.

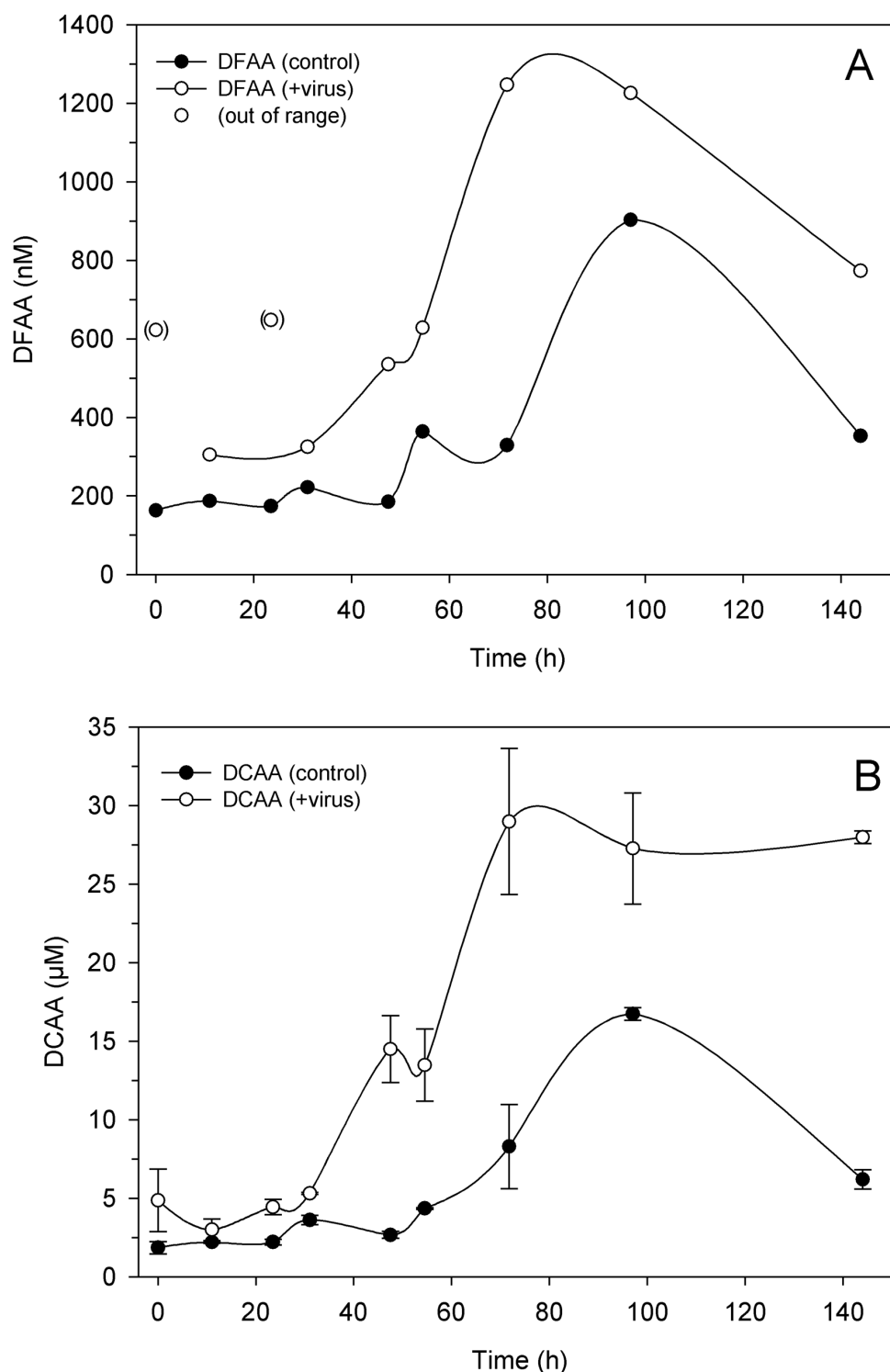


Figure 2. (A) Concentration of dissolved free amino acids (DFAA) and (B) dissolved combined amino acids (DCAA) in the control and +virus cultures. For DFAA single concentrations are shown, while concentrations of DCAA are means \pm SD (N=3). The two data points in parentheses in (A) are considered outliers.

analysis were filtered through 0.2 μ m Minisart membrane filters (www.sartorius.com) and frozen immediately for later analysis.

Microbiological and chemical analyses

Viruses and bacteria were quantified according to Noble & Fuhrman (1998). Subsamples of 0.5–2 ml were filtered onto 0.02 μ m Anodisc filters (Whatman), placed on a

drop of 0.2% SYBR-Green I (Molecular Probes) for 15 min and mounted on a glass slide. Three hundred to 600 bacteria and viruses were counted on each slide by epifluorescence microscopy.

Dissolved free and combined amino acids (DFAA and DCAA) were quantified by high performance liquid chromatography (HPLC) and fluorescence detection using two methods. Total amounts of DCAA (after hydrolysis, see below) and DFAA were detected as fluorescent primary

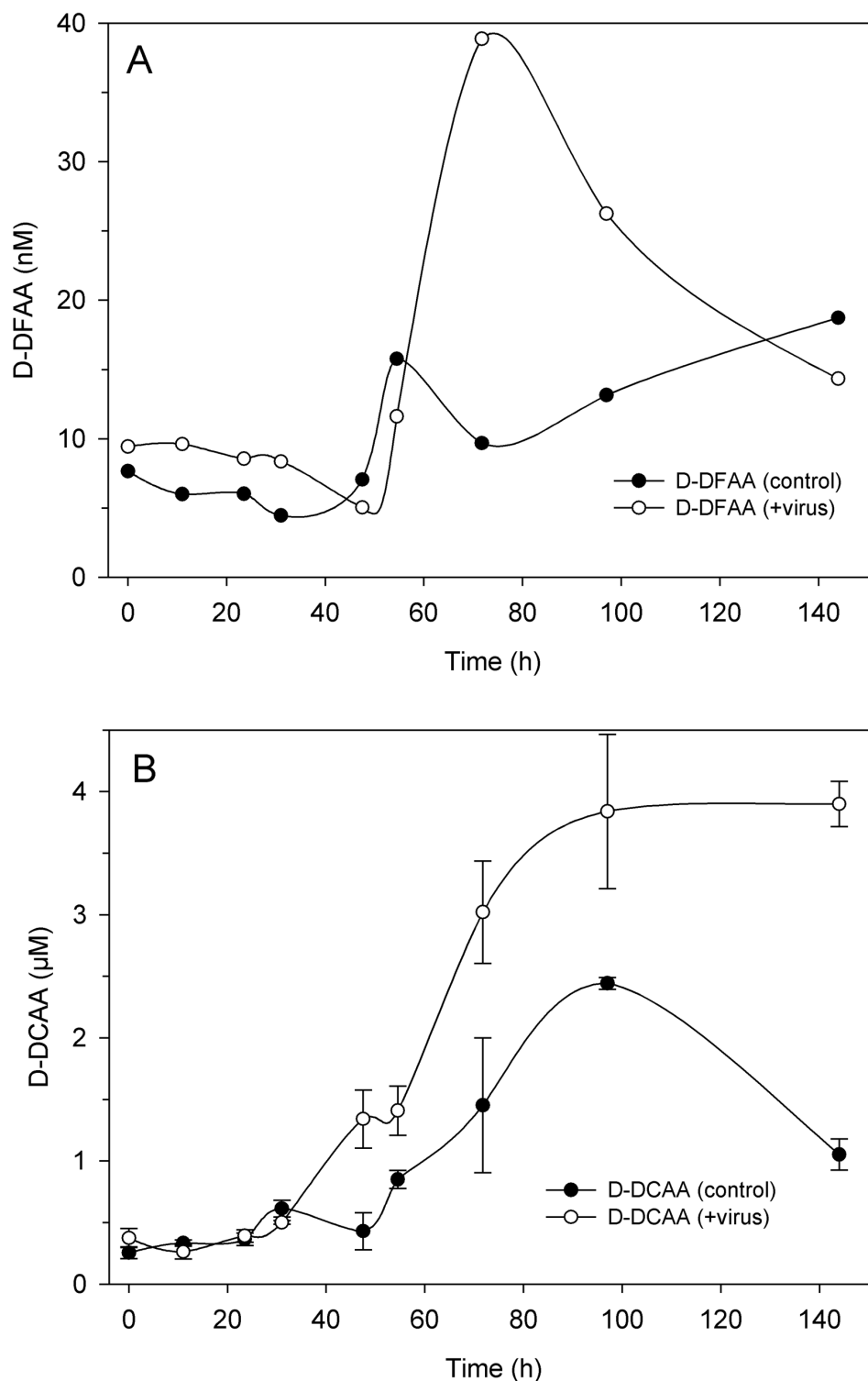


Figure 3. (A) Concentration of the cell wall derived D-isomers of dissolved free amino acids (D-DFAA) and (B) dissolved combined amino acids (D-DCAA) in the control and +virus cultures. For DFAA single concentrations are shown, while concentrations of DCAA are means \pm SD (N=3).

amines after derivatization with *o*-phthalaldehyde (OPA) according to Lindroth & Mopper (1979) and Jørgensen et al. (1993). In addition to protein amino acids (not including Pro and Cys due to lack of reaction with OPA), the detection included ornithine (Orn), α -aminobutyric acid (α -ABA), γ -aminobutyric acid (GABA), and *m* and LL-isomers of diaminopimelic acid (DAPA). For the analysis of DCAA, triplicate water samples were freeze-dried and subsequently hydrolysed by a microwave

technique. The hydrolyzed samples were re-dissolved in 1.25 M borate buffer adjusted to pH 9.5, filtered through 13 mm diameter 0.2 μ m pore size filters and analysed by HPLC. Concentrations of DFAA were subtracted from the concentrations of total dissolved hydrolysable amino acids to provide DCAA concentrations. All DFAA concentrations are presented as single concentrations as the variation between replicate analyses (injections) was within the method (machine) variability of maximum 5%.

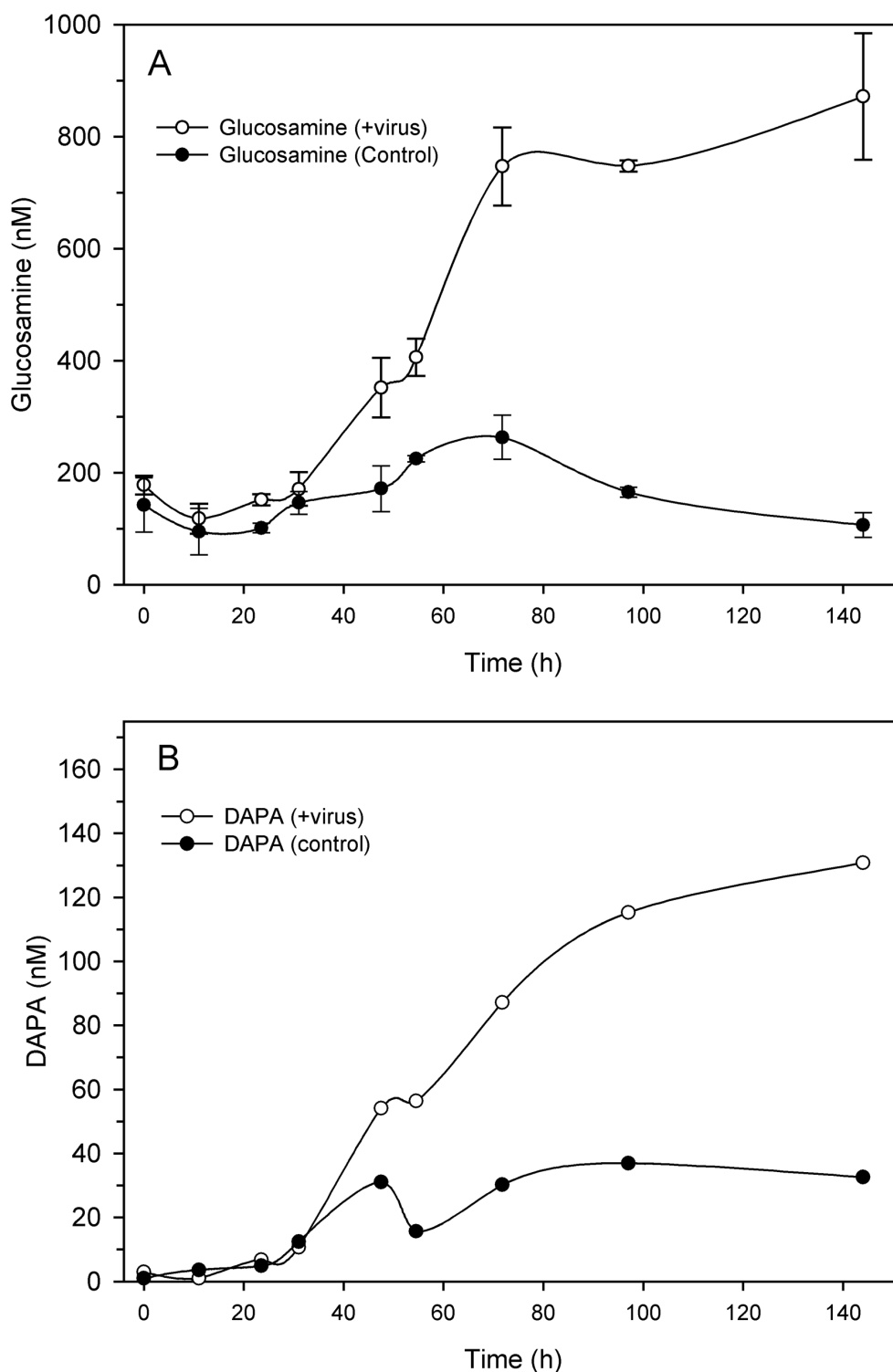


Figure 4. (A) Concentration of the cell wall derived compounds glucosamine and (B) diaminopimelic acid (DAPA) in the control and +virus cultures. Concentrations of glucosamine are means \pm SD (N=3), while DAPA is shown as single concentrations.

The D and L isomers of Asp, Glu, Ser and Ala were measured by the method of Mopper & Furton (1999) with the exception that N-isobutyryl-L-cysteine (IBC) was used as a chiral agent (Brückner et al., 1994). The HPLC columns used were 3.9×150 mm steel columns, type Nova-Pak C18 (for the OPA method) and XTerra RP18 (for the IBC method) (Waters Associates, USA). The detection of amino acid D isomers focused on Asp, Glu, Ser and Ala as these amino acids have been found to

be dominant isomers in natural and biological material, including bacterial peptidoglycan (Brückner et al., 1994; Jørgensen et al., 2003). The D isomer detection of amino acids also allowed a quantification of glucosamine in the DCAA fraction (glucosamine only occurred at trace concentrations in the DCAA pool). Glucosamine was detected in all the DCAA samples and was assumed to originate from N-acetyl-glucosamine in peptidoglycan. Probably the acid hydrolysis removed the acetyl groups.

Table 1. *Estimated release of organic carbon as viral lysates and the specific contribution of DFAA, DCAA, glucosamine and viral particles to the lysates. Concentrations of DFAA, DCAA and glucosamine and contributions to the lysate production are net (see text).*

Total lysate release (0–71 h) ($\mu\text{g C l}^{-1}$)	Virus mediated release of specific compounds (0–71 h) ³							
	DFAA ($\mu\text{g C l}^{-1}$)	% of total lysates	DCAA ($\mu\text{g C l}^{-1}$)	% of total lysates	Glucosamine ($\mu\text{g C l}^{-1}$)	% of total lysates	Viral particles ⁴ ($\mu\text{g C l}^{-1}$)	% of total lysates
1078 ¹ –1875 ²	31	2–3	924	51–86	33	2–3	67	4–6

¹, Estimated from the production of viruses and assuming a burst size of 50; ², estimated as the difference in bacterial biomass produced in control and +virus cultures; ³, estimated from the difference in concentrations between +virus and control cultures; ⁴, assuming a virus carbon content of 0.2 fg virus⁻¹ (Suttle, 2005).

RESULTS

Densities of bacteria and viruses

Bacterial abundance in the control cultures (without viruses) increased from the initial 0.2×10^6 cells ml⁻¹ to approximately 1×10^7 cells ml⁻¹ after 55 h (Figure 1). In the cultures with addition of viruses (+virus), bacterial density decreased from an initial density similar to that in the control, to about 0.07×10^6 cells ml⁻¹ after 31 h, followed by an increase to 6.2×10^6 cells ml⁻¹ at 144 h. The density of viruses increased from 0.1×10^6 virus particles ml⁻¹ to a level of about 3×10^8 ml⁻¹ after 54 h. No virus production was detected in the control cultures.

Amino acid concentrations

The total DFAA concentration in the control cultures increased from a level of about 200 nM during the initial 48 h to 900 nM at 97 h, followed by a decline to 350 nM at 144 h (Figure 2A). In the virus-enriched cultures the DFAA concentration was slightly higher than in the control (300–400 nM) during the initial 31 h, probably due to the addition of the virus stock solution (Figure 2A). The concentration then increased to about 1250 nM at 72 h (17 h after the viral density reached its plateau), but declined subsequently to 775 nM at 144 h (Figure 2A).

The DCAA pool showed a similar temporal development in the two treatments. The DCAA concentration increased over time in all cultures and reached a maximum of 17 μM in the controls (after 97 h) and a level of 25 to 30 μM in the +virus cultures (after 71 h) (Figure 2B). In the controls, the DCAA concentration decreased during the last part of the incubations.

Concentrations of the four analysed D-isomers were lower than L-isomer concentrations in all the cultures. D-DFAA concentrations varied in the controls from 4 to 7 nM until 48 h, and then increased to 19 nM at 144 h (Figure 3A). Relative to D-DFAA, the L-DFAA concentrations of the four analysed amino acids were 19- to 50-fold higher. In the +virus cultures, changes in the D-DFAA concentration reflected the pattern of total DFAA, but with 32- to 106-fold lower concentrations (Figures 2A & 3A). Here, D-DFAA concentrations increased from 5–9 nM to 39 nM at 72 h, and finally declined to 14 nM at 144 h.

In the DCAA pool, D isomer concentrations in the controls and +virus cultures reflected the changes observed in the total DCAA pools in these cultures (Figures 2B & 3B). From rather stable initial levels of

300–400 nM, D-DCAA concentrations increased to maximum values of 2.4 μM and 3.9 μM in the control and +virus cultures, respectively (Figure 3B). As for the total DCAA, the D-DCAA declined during the last 50 h in the controls. In the virus-enriched cultures, the proportion of D isomers, relative to all DCAA, increased from 16–18% during the first 72 hours, to 25% at 97 and 144 hours. In contrast, the proportion of D isomers was relatively unchanged in the control cultures and made up $29\% \pm 3$ of all the DCAA.

Production of peptidoglycan-specific amino acids

The production of viruses also affected the concentrations of glucosamine. In the control cultures, glucosamine ranged from 95 to 145 nM up to 31 h where the concentration slowly increased to a maximum of 265 nM at 72 h (simultaneous with the first peak in bacterial density). Subsequently, the concentration declined to 105 nM and did not reflect the bacterial peak at 144 h (Figure 4A). In the +virus cultures, initial glucosamine concentrations were slightly higher (120–170 nM up to 31 h), but reached a level of 750 nM at 72 h (17 h after the viral density reached its plateau). Finally, glucosamine concentration increased to 875 nM at 144 h (Figure 4A).

In the DFAA pool, DAPA (amino acid in the peptidoglycan interbridges in Gram-negative bacteria; sum of *m* and LL isomers are shown) increased in the control cultures from initially <12 nM to a level of 30–37 nM during the incubation, except for a decline to 16 nM at 55 h (Figure 4B). In the +virus cultures, on the other hand, the DAPA concentration increased during incubation to a final level of 130 nM, or four-fold higher than in the control cultures (Figure 4B). The DAPA could not be quantified in the DCAA pool due to interference (co-elution) from an unknown amino acid.

DISCUSSION

Viral lysis of bacteria is known to release bacterial cell content and produce cell wall debris, but the specific chemical composition and bioavailability of the lysates are largely unknown. Potentially the lysates constitute a valuable source of nutrients to other bacteria and may hereby function as readily re-assimilated compounds. Here we present for the first time an analysis of the organic amino compounds being released during viral lysis. The results demonstrate that viral lysates are significant sources of highly labile dissolved organic material

that can be utilized by other bacteria, including cell wall components (D amino acid isomers, glucosamine and diaminopimelic acid (DAPA)) as well as dissolved free and combined amino acids.

Viral control of bacterial growth

Addition of viruses had a pronounced effect on the bacterial density in the cultures. In contrast to the control cultures, where the cell density had reached 4×10^6 cells ml^{-1} after 31 h, the addition of viruses completely eliminated any accumulation of cells during that period. Following lysis of sensitive *Cellulophaga* cells, the population was replaced by virus-resistant clones of the same bacterial strain (tested by re-infection experiments of selected colonies isolated from the cultures; data not shown). This change in bacterial clonal composition following viral lysis has been observed in several other studies (e.g. Middelboe et al., 2003), and emphasizes the potential importance of the dynamics of specific clones of a given bacterial population in response to viral infections of bacterial populations.

Virus-induced disintegration of cell wall peptidoglycan

In the control cultures, release of dissolved amino compounds peaked during the stationary growth phase, coinciding with a temporary decrease in cell numbers between 71 and 100 h. This suggested that the released amino compounds originated from the disintegration of bacterial cells. However, concentrations of the analysed compounds were significantly higher in the presence of viruses, indicating that viral activity was the major cause of the released amino compounds. The difference in amino compounds between the control and the virus-enriched cultures may be used as an indicator of the effect of virus infection on organic matter production due to viral infection (see below).

The significant production of D amino acids, glucosamine and DAPA demonstrated that peptidoglycan components were released as part of the viral lysates. These compounds originate from bacterial cell walls and may have been released from peptidoglycan-hydrolysing enzymes when viruses penetrated the cells (Rydman & Bamford, 2002) or as cell debris following lysis.

Mass balance of viral lysis

The applied model system of *Cellulophaga* sp. and its specific virus has previously been used to demonstrate that virus-mediated *Cellulophaga* sp. lysates are efficiently utilized by a co-occurring, non-infected bacterial population (*Photobacterium* sp.) during similar experimental conditions (Middelboe et al., 2003). In these experiments the release of organic carbon during cell lysis was estimated from the decrease in numbers of *Cellulophaga* sp., their average cell volume, and a theoretical carbon:volume conversion factor. In the present study, we could not directly determine the number of lysed cells from the decrease in cells numbers, since there was no accumulation of bacteria in the cultures prior to viral lysis. We have therefore presented the number of lysed cells as a range of values, which are derived from two different calculations of the loss of cells during incubation (0–71 h): the maximum value was obtained as the difference in cell numbers between the control and +virus

cultures, corresponding to 1.0×10^7 cells ml^{-1} . However, since viral lysis may have limited cell production in the +virus cultures, this may be an overestimate. We have therefore also estimated the number of lysed cells from the production of viruses and assuming virus burst size of 50, which resulted in an estimated 6.7×10^6 cells ml^{-1} being lysed by viruses between 0 and 71 h. This latter calculation represented the minimum value of cell lysis, although the use of a higher burst size value would have led to a lower number of lysed cells. The reason for that is that a lower value of cell lysis could not explain the observed amount of amino acids released in the cultures (see below). We believe therefore, that the range from 6.7×10^6 to 1.0×10^7 lysed cells ml^{-1} represent a reasonable estimation of the impact of viruses on cell lysis in the cultures. Using the empirically determined cell volume for *Cellulophaga* sp. given in Middelboe et al. (2003) and a theoretical carbon conversion factor of $0.31 \text{ pg C } \mu\text{m}^{-3}$ (Fry, 1990), the total organic carbon release was estimated to $1078\text{--}1875 \text{ } \mu\text{g C l}^{-1}$ (Table 1).

Combined amino acids constituted the major fraction of the released dissolved material. Based on the amino acid carbon contents, the DCAA input constituted 924 g C l^{-1} or 51–86% of the total C-release (Table 1). The DFAA and glucosamine inputs were 31 and 33 g C l^{-1} , respectively, each corresponding to 2–3% of the lysates. The viral particles themselves also contributed to lysate-DOM (Table 1). Assuming a carbon content of $0.2 \text{ fg virus}^{-1}$ (Suttle, 2005), the produced viruses amounted to $67 \text{ } \mu\text{g C l}^{-1}$ or 4–6% of the lysate-C (Table 1). However, since viruses consist mainly of proteins, most of this carbon was probably already accounted for in the DCAA analyses. In contrast to these virus-rich lysates, the general contribution of virus proteins to the bulk dissolved amino acid pool in marine aerosols have recently been found to be insignificant ($<10 \text{ nM}$) (Kuznetsova et al., 2005). When all the chemical measurements were pooled, we could account for 59–92% of the released organic matter, and of this about 12% appeared to originate from bacterial cell wall peptidoglycan (D-AA, glucosamine and DAPA). The remaining, unidentified fraction of the lysates probably consists of particulate ($>0.2 \text{ } \mu\text{m}$) cell debris, polysaccharides and nucleic acid material. The calculated estimates of lysate products relative to the viral density must be considered as net (or minimum) values, since an uptake of cell lysates by actively growing bacteria occurred during the incubation as discussed below.

Bacterial uptake of viral lysates

Bacterial cell wall material has often been regarded as refractory to bacterial decomposition relative to bacterial proteins (e.g. Nagata et al., 2003), but other studies indicate that peptidoglycan D amino acid isomers may constitute an important nutrient source for bacteria under nutrient limited conditions (e.g. Perez et al., 2003). Here, we also found significant bacterial uptake of D isomers during the last stage of the incubations: in the control culture, 63% of the released D-DCAA was taken up during the last 50 h, concomitant with a secondary increase in bacterial abundance. In the +virus culture, 83% of the released D-DFAA was utilized again during regrowth of virus-resistant *Cellulophaga* clones. From previous studies (Middelboe et al., 2003) we know that

Cellulophaga are not able to take up glucosamine, which may explain the accumulation of that compound in the cultures. In general, however, our data suggest that viral lysis can be a significant source of D amino acids and other cell wall derived compounds in marine environments, and that a portion of these compounds is efficiently recycled by free-living bacteria.

The biomass of resistant *Cellulophaga* that were produced during the last part of the incubations was estimated from the increase in cell numbers, using cell volume and a carbon conversion factor given in Middelboe et al. (2003). The resistant *Cellulophaga* cells are smaller than the wild type cells and have an average volume of $0.30 \mu\text{m}^3$ (Middelboe et al., 2003). That way the net production of virus-resistant cells was determined to $450 \mu\text{g C l}^{-1}$. The concomitant net uptake of DCAA and DFSA of 49 and $23 \mu\text{g C l}^{-1}$, respectively, could account for only 16% of the produced biomass, suggesting that growth of resistant *Cellulophaga* may primarily have been sustained by a fraction of the initially added glucose, which was left unused by the virus-controlled sensitive population.

Conclusions

The contribution of viral-mediated organic matter obtained in this study was probably higher than might be expected at natural conditions, since the cultured cells were large relative to most natural bacterioplankton cells. On the other hand, it is important to stress that lysates may contribute substantially to the global DOM pool as a significant fraction of pelagic bacterial production is released as viral lysates. In that perspective it is crucial to obtain information about the amount, composition, and bioavailability of these lysates. Our data provided such information in an experimental bacteria-virus system and demonstrated that viral lysates may indeed contribute to different pools of amino acids and cell wall-derived compounds. A portion of this cell wall material probably enters the pool of refractory material, but up to 83% of specific amino acid compounds were rapidly recycled by non-infected bacteria. With the high N content of the released compounds, relative to the optimum C:N ratio in bacterial substrates, it is reasonable to believe that a large fraction of amino-N in the lysates will be mineralized to bioavailable ammonium. Overall, our study supports the prevailing assumption that viral transformation of particulate organic matter (living cells) to dissolved compounds has significant implications for the cycling of organic matter and nutrients in the ocean.

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